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Characterization of membrane protein reconstitution in LUVs of different lipid composition by fluorescence anisotropy

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ABSTRACT

A major requirement to perform structural studies with membrane proteins is not only to define efficient reconstitution protocols, that assure a high incorporation degree in preformed liposomes, but also a protein directionality and topology that mimics its *in vivo* conditions. For this kind of studies, protein reconstitution in membranes systems *via* a detergent-mediated pathway is usually successfully adopted, since detergents are generally used in the initial isolation and purification of membrane proteins. In this study we report the reconstitution of OmpF in preformed DMPC and *E. coli* liposomes using two different techniques for detergent removal: (1) exclusion chromatography and (2) incubation with detergent-adsorbing beads. The incorporation degree was determined by bicinchoninic acid assay and fluorescence anisotropy was used to determine OmpF effect on the structural order of membrane lipids. These results show that protein insertion in membranes depends both on the technique used to remove detergent and on the lipids used to prepare the liposomes. Furthermore, it is possible to state that although the insertion is directly related to the size distributions of proteoliposomes, it could be efficiently recognized by steady-state fluorescence anisotropy. This technique, more popular among cell biologists, can be a very practical and straightforward alternative to DLS to confirm membrane protein insertion.

1. Introduction

Membrane proteins constitute an important research area due not only to their role in control of fundamental biochemistry processes, but also to their importance as pharmaceutical targets [1]. However they are generally not soluble in aqueous solution and require special synthetic systems for *in vitro* work. Several experimental methods have been devised for the study of these proteins *in vitro*, taking into account their location in environments that satisfy their higher hydrophobicity [1–3].

Proteoliposomes, lipidic vesicles in which membrane proteins are inserted, are model membranes systems often used for elucidation of membrane protein structure and function. However, for them to be useful a number of conditions should be met, the main ones being: a homogeneous size distribution, an even distribution of protein among liposomes and a system that mimics the *in vivo* conditions [4].

A variety of methods can be used to insert membrane proteins in liposomes but for structural studies reconstitution *via* a detergent-mediated pathway is usually successfully and conveniently used, as detergents are usually used in the initial isolation and purification of membrane proteins [5–8]. One approach, commonly used, is the introduction of protein/detergent into preformed liposomes in a way that the liposome bilayer becomes saturated with detergent, disrupting lipid–lipid interaction and resulting in a more permeable and receptive bilayer to protein uptake, followed by removal of detergent by one of several techniques (dialysis, column exclusion chromatography or incubation with detergent-adsorbing beads) [1–3].

OmpF is an outer membrane protein that is present in several bacteria strains and seems to plays an important role in the uptake of several different families of antibiotics [9,10]. Nevertheless, it is still unknown if the entry of the antibiotics is through the lipid/protein interface or through the porin channel [10,11]. The study of a possible interaction of antibiotics with OmpF protein in different model membranes is important, not only to understand the relationship between the structural properties of the protein and the uptake of these drugs, but also to try to relate antibiotic resistance, in a quantitative manner, to translocation across the outer membrane. To perform these structural studies with OmpF it is mandatory to define efficient reconstitution protocols and the first step must be to assure a high incorporation degree in preformed liposomes and a protein directionality and topology that mimics *in vivo* conditions.

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In this study we report the reconstitution of OmpF in preformed liposomes of DMPC and *E. coli* lipids using two different techniques for detergent removal, exclusion chromatography and incubation with detergent-adsorbing beads. The incorporation degree was determined by using bicinchoninic acid protein assay and fluorescence anisotropy was used to determine the effect of OmpF on the structural order of membrane lipids. Dynamic light scattering was used as a complementary technique to validate fluorescence anisotropy. The results obtained show that the incorporation degree does not depend on the reconstituting methodology or on the lipid used on the preformed liposomes. Nonetheless, OmpF effect on the structural order of membrane lipids depends not only on the technique used to remove detergent but also on the lipid used to prepare the preformed liposomes. This effect, although directly related to the size distributions of proteoliposomes, can be efficiently determined by steady-state fluorescence anisotropy.

2. Materials and methods

N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid (HEPES) was from Sigma (Sigma, St. Louis, MO). Octylpolyoxyethylene (oPOE) was from Bachem (Bubendorf, Switzerland), 1,6-diphenyl-1,3,5-hexatriene (DPH) and trimethylammonium-diphenylhexa-triene (TMA-DPH) were from Sigma, L-α-dimyristoylphosphatidylcholine (DMPC), *E. coli* total extract lipids (*E. coli*) were from Avanti Polar Lipids, all other chemicals from Merck (Darmstadt, Germany). All solutions were prepared with 10 mM HEPES buffer (0.1 M NaCl; pH 7.4). OmpF was purified from *E. coli*, strain BL21 (DE3) Omp8, following published procedures [12,13]. OmpF concentration was estimated using the bicinchoninic acid protein assay against bovine serum albumin as standard [14,15]. All the fluorescence measurements were performed in a Varian spectrofluorometer, model Cary Eclipse, equipped with a constant-temperature cell holder (Peltier single cell holder).

2.1. Liposome preparation

Chloroform/methanol (1:1, v/v) solutions (*E. coli* lipids) and chloroform solutions (DMPC) containing the appropriate amount of lipids were dried under a stream of oxygen-free argon in a conical tube. The thin film obtained was kept under high vacuum for more than 3 h to remove organic solvent traces. Multilamellar liposomes (MLVs) were obtained after redispersion of the film in 10 mM HEPES buffer (0.1 M NaCl, pH 7.4) and vortexed above the phase transition temperature (37 ± 1 °C). Frozen and thawed MLVs were obtained by repeating five times the following cycle: freezing the vesicles in liquid nitrogen and thawing the sample in a water bath at 37 ± 1 °C. Suspensions of MLVs were then stabilized at 37 °C for 30 min and extruded 10 times, on a Lipex Biomembranes (Vancouver, Canada) extruder attached to a circulating water bath through polycarbonate filters (100 nm) to produce large unilamellar vesicles (LUVs). Lipid concentration in vesicle suspensions was determined by phosphate analysis, using a modified version of the Fisk and Subbarow method [16].

2.2. Protein reconstitution

OmpF proteoliposomes were prepared by direct incorporation into preformed DMPC or *E. coli* liposomes, using two different techniques for detergent removal [2,4,17,18], reconstitution of OmpF protein in *E. coli* lipids bilayer and DMPC liposomes using detergent adsorption on polystyrene beads (Bio-Beads SM-2, Bio-Rad) and reconstitution of OmpF protein in the same liposomes using gel exclusion chromatography (Sephadex G50).

In the first technique a determined quantity of OmpF (0.11 mg/ml) in a HEPES buffer solution with 0.4% of oPOE is added to an adequate volume (~2.6 ml) of DMPC or *E. coli* LUVs (~2.0 mM) in HEPES buffer. The mole ratio between lipid/protein was always near 1000 and the total volume of the mixture assures a final concentration of oPOE lower than value of detergent critical micelle concentration (CMC)—0.23%. After a good homogenization of all constituents by gentle stirring, the mixture was incubated 15 min at room temperature followed by 1 h on ice. The detergent was then adsorbed onto SM2 Bio-Beads at a concentration of 0.2 g of Bio-Beads/ml, by gentle shaking of the suspension during a period of 3 h. After this time, a second portion of the same amount of Bio-Beads was added and the suspension was again shaken for another 3 h. At the end of this period, proteoliposomes were gently removed by decanting the Bio-Beads. The proteoliposomes were collected by ultracentrifugation for 150 min at 80,000 × *g* (4 °C), and resuspended in a small volume of 10 mM HEPES buffer. The suspension was then extruded 5 times through polycarbonate filter (200 nm) and 10 times for 100 nm filter at 37 °C.

In the second method the LUVs were mixed with solubilized protein OmpF to a final lipid-to-protein ratio of 1000:1 (mol/mol) as before. The mixture was incubated at room temperature for 30 min with gentle agitation and applied to a column of Sephadex G50, equilibrated at room temperature, with 10 mM HEPES buffer (0.1 M NaCl, pH 7.4). Fractions of about 1 ml were recovered in the same buffer, at a flow rate of 0.5 ml/min and proteoliposomes were collected in volumes of approximately 3 ml. For both techniques liposomes alone were treated in a similar way.

2.3. Steady-state anisotropy experiments

In this study the effects of OmpF on the structural order of lipid membrane were investigated by measuring, as a function of temperature, the steady-state fluorescence anisotropy (r_s) of DPH (1,6-phenyl-1,3,5-hexatriene) and TMA-DPH (1-(4-trimethylammoniumphenyl)- 6-phenyl-1,3,5-hexatriene) incorporated into liposomes and proteoliposomes of *E. coli* and DMPC phospholipids. The suspensions of liposomes and proteoliposomes were mixed with TMA-DPH and DPH for 30 min at 37 °C, to a final lipid-to-fluorescent probe ratio of 300:1 (mol/mol). The excitation wavelength was 355 nm for TMA-DPH and 336 nm for DPH. The emission was measured at 426 and 427 nm for TMA-DPH and DPH. The anisotropy was recorded at 5° intervals (*E. coli*) and at 3° intervals (DMPC) in the range 0–40 °C.

2.4. Dynamic light scattering

The size distribution of the liposomes and proteoliposomes was determined by dynamic light scattering analysis using a Malvern Instruments (Malvern, UK) Zeta Sizer Nano ZS.

2.5. Atomic Force Microscopy (AFM) experiments

Samples for AFM were prepared by depositing a 20 μl volume of the proteoliposome solution in HEPES buffer on freshly cleaved mica at room temperature. 20 min was allowed for the vesicles to fuse, and then the sample was washed three times with the same buffer, finally leaving a volume of *ca.* 15 μl in which to image. Imaging was carried out at room temperature in buffer using tapping mode. NP-S probes with a spring constant of approximately 0.06 N/m (Veeco, California, USA) were used at an oscillation frequency of approximately 8 kHz. All imaging was carried out with multimode AFM and Nanoscope IVa electronics (Veeco). In all cases, the presence of the lipid bilayer (which was featureless)

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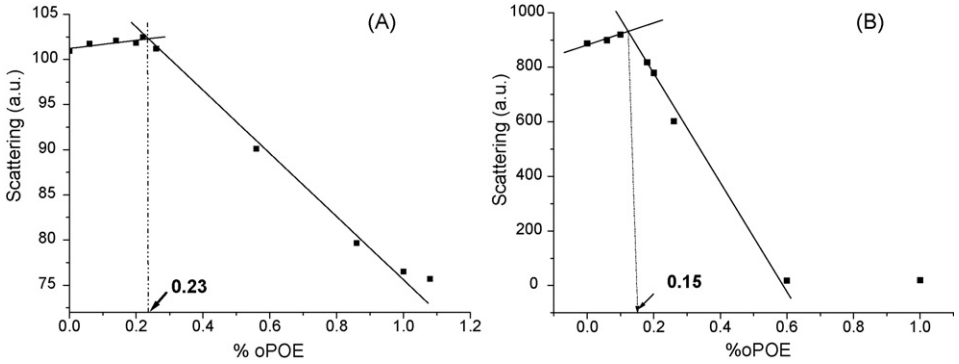

Fig. 1. Scattering changes measured in solutions of (A) DMPC LUV and (B) *E. coli* LUV in the presence of increasing concentrations of oPOE (0–1.1%).

was verified by pushing the AFM tip through the layer using the breakthrough technique described in [19].

3. Results

OmpF has a very high stability in oPOE micellar solution, and most of the purification procedures described in literature for this protein are performed in this detergent [13]. So, in order to develop procedures for OmpF reconstitution, in preformed liposomes, the first step was the determination of the oPOE concentrations that can give rise to mixed micelles by optimization of the oPOE/liposome ratio. The results depicted in Fig. 1 show that the transition to a micellar state begins for DMPC at oPOE CMC (0.23%) and for *E. coli* liposomes at a concentration a little smaller than the oPOE CMC (0.15%). Dynamic light scattering determinations performed on these solutions corroborate these results (data not shown).

After reconstitution, OmpF's concentration was determined in the proteoliposomes (see Section 2) and the results show that the incorporation degree is independent of the techniques used for detergent removal. However the incorporation degree is always higher for *E. coli* liposomes, with 92% value for this lipid extract and was 85% for DMPC. Although a high incorporation degree was obtained for the different used methods, steady-state fluorescence anisotropy studies and light scattering determinations were performed in order to know if the protein directionality and topology is the one that better mimics the *in vivo* conditions.

3.1. Steady-state fluorescence anisotropy

All the reconstitution procedures were accompanied by steady-state fluorescence anisotropy studies of DPH and TMA-DPH probes inserted on liposomes and proteoliposomes. DPH is embedded in the bilayer while TMA-DPH is anchored at the aqueous interface, with its DPH moiety intercalated between the phospholipid acyl chains. Consequently, it is possible to gather information on the protein incorporation into the core and/or at the interface regions of the bilayer.

The steady-state fluorescence anisotropy (r) is defined by the following equation [20]:

$$r = \frac{I_{VV} - I_{VH}G}{I_{VV} + 2I_{VH}G} \quad (1)$$

where I_{VV} and I_{VH} are the intensities measured in directions parallel and perpendicular to the excitation beam. The correction factor G is the ratio of the detection system sensitivity for vertically and horizontally polarized light, which is given by the ratio of vertical to horizontal components when the excitation light is polarized in the horizontal direction, $G = I_{HV}/I_{HH}$ [21].

The temperature dependence of DPH and TMA-DPH anisotropy in DMPC liposomes and proteoliposomes, for the two techniques used for detergent removal, are depicted in Fig. 2. The following equation was fitted to the anisotropy versus temperature data

$$r_s = r_{s2} + \frac{r_{s1} - r_{s2}}{1 + 10^{B'(T/T_m - 1)}} \quad (2)$$

where T is the absolute temperature, T_m is the midpoint phase transition and r_{s1} and r_{s2} are the upper and lower values of r_s; B' is the slope factor which is correlated with the extent of cooperativity (B) by $B = [1 - 1/(1 + B')]$; the introduction of B yields a convenient scale of cooperativity ranging from 0 to 1 [22].

A glance at these results show that the anisotropy profiles for both probes in liposomes and proteoliposomes are clearly different. For the DMPC liposomes the anisotropy values suffer an abrupt change in the transition temperature region, giving a mean $T_m = 24.4 \pm 0.03\,^{\circ}C$. For proteoliposomes, however, the profiles are quite different showing a decrease in anisotropy values below the T_m and an increase after the T_m, than the ones observed for pure lipids. This is not an unexpected value since the insertion of a membrane protein is known to alter membrane fluidity properties.

However, the T_m values found for the proteoliposomes were only slightly lower than those found for the liposomes. A T_m of $23.6 \pm 0.01\,^{\circ}C$ and of $23.0 \pm 0.05\,^{\circ}C$ is obtained for DPH and TMA-DPH, respectively. Moreover, these values were independent of the detergent-removal method.

A closer look of Fig. 2 also shows that the anisotropy profiles for the TMA-DPH probe (B and D) are identical, within experimental error, for the two techniques used for detergent removal, but the same is not straightforward, for the DPH probe (A and C). In fact, the DPH probe anisotropy profile in proteoliposomes (when the column method approach is used for detergent removal) was almost identical to the profile obtained for liposomes. This is indicative that OmpF insertion did not affect the core region of the liposomes where the DPH is localized [23]. If the protein was inserted in the proteoliposomes (by the column method), as expected, it would cross the lipid membrane from one side to the other changing the DPH anisotropy profile.

Changes in anisotropy values above phase transition were identical for both techniques, $\Delta r \sim 0.05$, for TMA-DPH, but for DPH this change is much higher when the detergent was removed by Bio-Beads, $\Delta r \sim 0.09$, than as it was removed by gel filtration, $\Delta r \sim 0.02$.

In Fig. 3, the temperature dependence of DPH and TMA-DPH anisotropy in *E. coli* liposomes and proteoliposomes, for both detergent-removal techniques, are represented. Eq. (2) was not fitted to these profiles since has been reported that *E. coli* lipid total extract has a small T_m [22] and it was not experimentally possible to go below this temperature. Analyzing the results it is possible to state that for the proteoliposomes prepared by detergent removal with Bio-Beads, there was an increase of anisotropy across all the temperature range ($\Delta r \sim 0.06$) and, detected for both probes. This increase in r_s values above T_m, observed for all proteoliposomes

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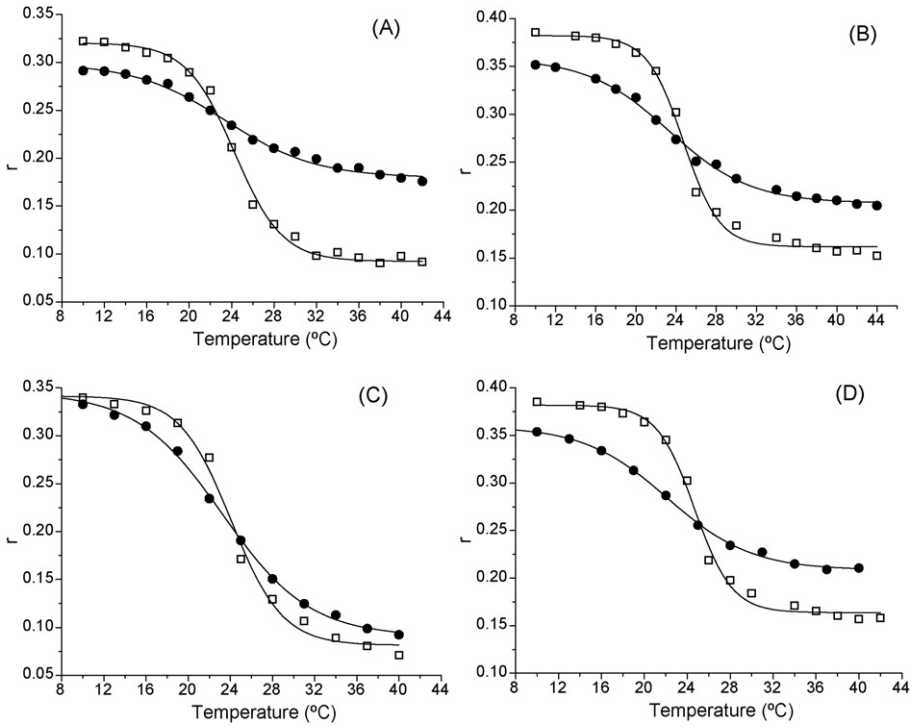

Fig. 2. Anisotropy change of the probe DPH (A and C) and TMA-DPH (B and D) in LUVs (-□-) and in proteoliposomes (-●-) of DMPC prepared by Bio-Beads detergent removal (A and B) and by gel filtration detergent removal (C and D). Results of at least three independent measurements.

prepared with Bio-Beads and for both probes, reflects an increase in molecular order which shows that the protein insertion was changing membrane properties, namely is making the lipidic fluid phase more ordered, as already observed for the insertion of several proteins and peptides in liposomes [24–27]. Nevertheless, when the proteoliposomes were prepared using the gel filtration approach to detergent removal, the anisotropy *vs.* temperature profile was similar for liposomes and proteoliposomes and furthermore for both probes.

The anisotropy profiles obtained are typical for liposomes and some proteoliposomes [22,25,26,28,29] except in the case of the *E. coli* proteoliposomes prepared by gel filtration. The outcome of

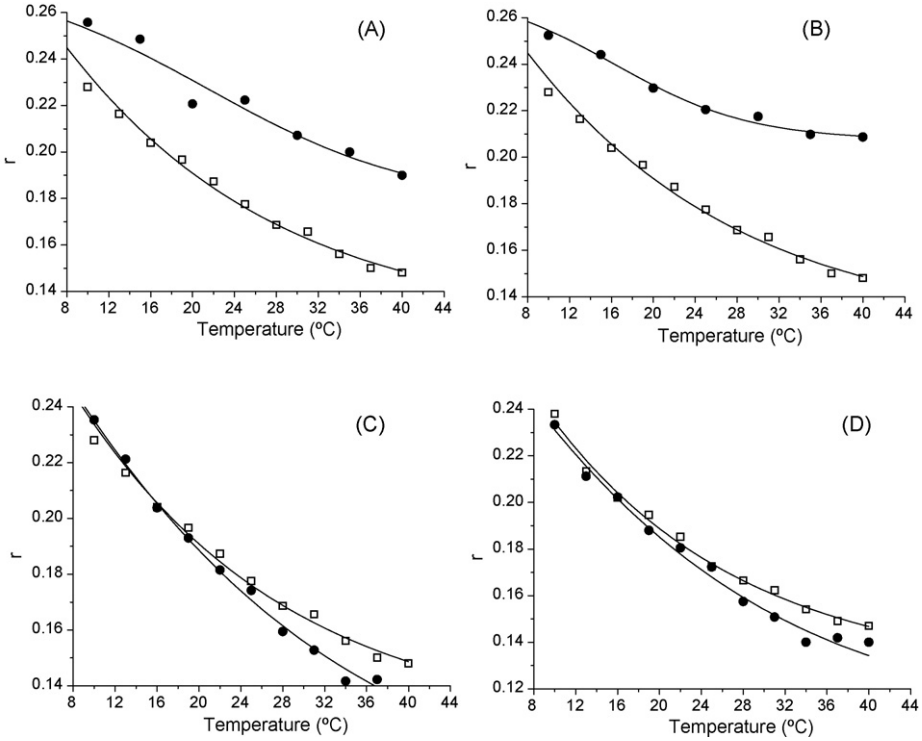

Fig. 3. Anisotropy change of the probe DPH (A and C) and TMA-DPH (B and D), in LUVs (-□-) and in proteoliposomes (-●-) of *E. coli* prepared by Bio-Beads detergent removal (A and B) and by gel filtration detergent removal (C and D). Results of at least three independent measurements.

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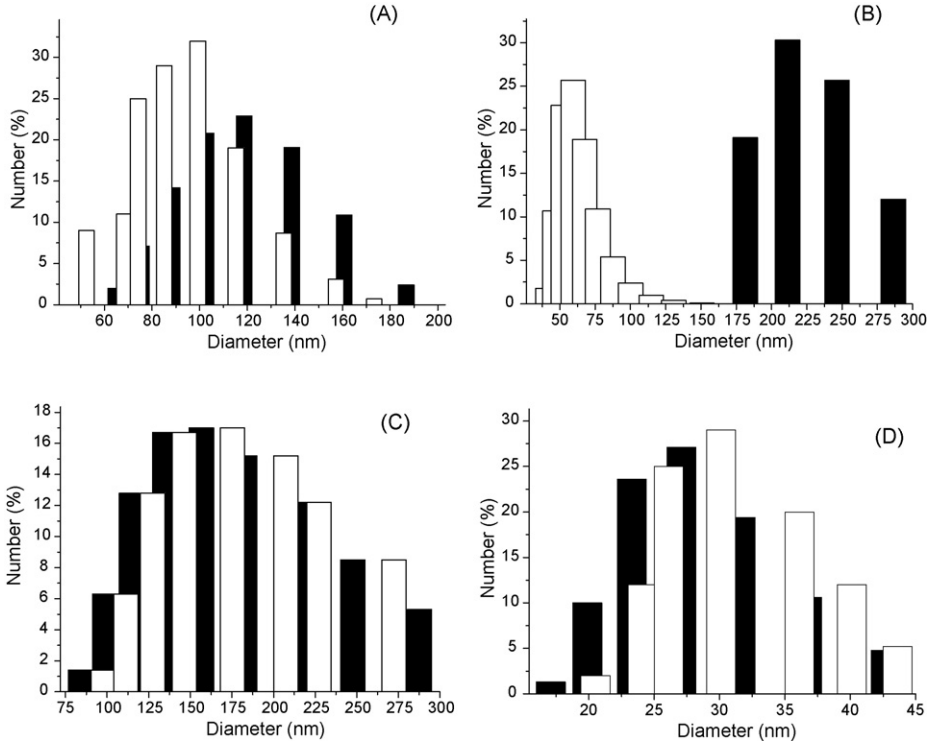

Fig. 4. Histograms of the size distribution of DMPC proteoliposomes prepared by (A) Bio-Beads detergent removal, (B) by gel filtration detergent removal and *E. coli* proteoliposomes prepared by (C) Bio-Beads detergent removal, and by (D) gel filtration detergent removal; columns for DPH (■), columns for TMA-DPH (□).

these results can only be attributed to a different protein insertion, since the probes could not sense a change in lipid fluidity, which preclude a correct protein insertion as OmpF is an integral protein that in its native conformation will change lipid order.

3.2. Dynamic light scattering

The size distribution of the liposomes, and proteoliposomes, were determined for all the samples used on the anisotropy experiments. The size of the liposomes as expected based on the procedure that was used for their preparation (see Section 2), show a mean diameter of 100.1 ± 4.5 nm for both lipids used. However, the results obtained for the size of proteoliposomes were more variable. As can be seen in Fig. 4, proteoliposomes prepared with the two lipids showed, always, two different profiles. For DMPC proteoliposomes it is possible to conclude that the size profile is the same for the proteoliposomes prepared with the two techniques used for detergent removal in the samples with TMA-DPH (diameter = 85.8 ± 20.1 nm) (Fig. 4A and B, □). However the size profile of the proteoliposomes prepared with DPH, by gel filtration detergent removal (Fig. 4B, ■), is quite different (diameter = 230.2 ± 71.4 nm). For *E. coli* proteoliposomes the size profile obtained with the two techniques used for detergent removal were rather different (Fig. 4C and D). The size profile obtained for the proteoliposomes prepared using the Bio-Beads detergent-removal approach is much broader than the size profiles obtained for the proteoliposomes prepared by gel filtration detergent removal. In addition the mean size of the proteoliposomes is much higher (155.1 ± 95.5 nm against 35.5 ± 13.0 nm).

These results are in agreement with those obtained by anisotropy, in that the *E. coli* proteoliposomes prepared by gel filtration and the DMPC proteoliposomes with DPH both showed an apparent difference in the protein insertion. Under these conditions, we found size profiles (either very small or very broad) that corroborated the differences seen in anisotropy measurements. We speculate that the relation between proteoliposome size and the insertion measurements by fluorescence anisotropy occur because the curvature stress between lipid and protein and bilayer elastic forces must be very different in these very small or very large proteoliposomes.

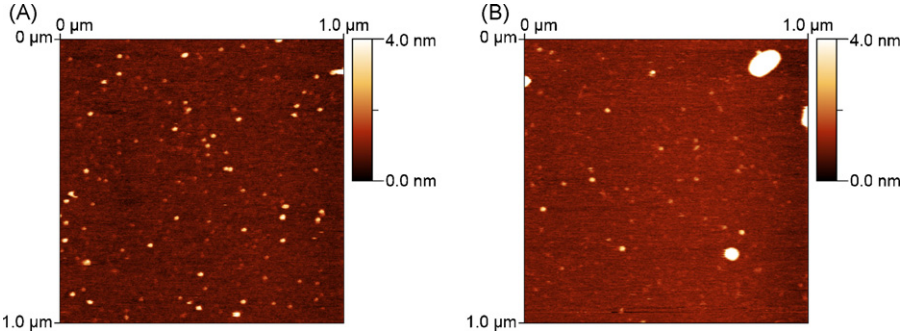

Fig. 5. AFM images recorded in buffer of supported lipid bilayers formed from proteoliposomes which were prepared by the Bio-Beads method (A) and by the gel filtration method (B).

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In addition AFM experiments were performed, in order to independently show the differences in the OmpF's reconstitution by the two methods. Fig. 5 shows AFM images of the supported lipid bilayers (SPBs) formed from the proteoliposomes as described. It was found that *via* the Bio-Beads method, the bilayers were very flat (see Fig. 5A) and homogeneous, showing only features consistent with inserted OmpF proteins (*i.e.* globular features of 1.8 ± 0.7 nm in height), and very occasionally protein aggregates. On the other hand, the layers formed from proteoliposomes which were reconstituted *via* the column method were very uneven," featuring many regions covered in multiple layers (e.g. the feature in the top right of Fig. 5B), and with vesicle-like structures adhered to the bilayer (e.g. the round feature towards the bottom right of Fig. 5B). The round features were proved to be lipidic in nature because occasionally these small vesicle-like features would collapse while imaging, leaving a second bilayer on top of the SPB. In places on the samples, these features almost covered the surface, making imaging consistent with visualizing single proteins impossible. The image shown in Fig. 5B was obtained on a relatively clean part of the sample. The samples produced *via* the column did also contain features consistent with inserted protein, again displaying globular features with a height of 1.7 ± 0.4 nm. The difference between the column and Bio-Beads produced samples was consistent across different samples and different lipid:protein ratios.

4. Discussion

To perform structural studies with proteoliposomes it is necessary to define efficient reconstitution protocols to assure a high incorporation degree in preformed liposomes and a protein directionality and topology that gives a high biological activity. As each protein has its special features it is important to develop analytical methodologies that can give, quickly and efficiently, information about the proteins directionality and topology to confirm that the higher biological activity was achieved.

In this work fluorescence anisotropy was used to determine the effect of OmpF on the structural order of membrane lipids using a reconstitution methodology in preformed liposomes of DMPC and *E. coli* lipids, but with two different techniques for detergent removal, exclusion chromatography and incubation with detergent-adsorbing beads. The anisotropy profiles achieved clearly show that insertion of the protein in DMPC liposomes was achieved by the two techniques and this result was confirmed by AFM, in presence of TMA-DPH, but for *E. coli* proteoliposomes it was only attained when detergent was removed by Bio-Beads. With these results it was possible to conclude that fluorescence anisotropy could be used as a technique to confirm correct insertion of OmpF in a conformation that can mimic its biological activity [30–32]. Nevertheless, to try to explain these results the size distribution of the proteoliposomes was determined by DLS and it was possible to conclude that the insertion was size dependent. This result is not surprising as reconstitution of membrane proteins not only depend on lipid composition but also, and more important, on hydrophobic mismatch and curvature stress between lipid and protein [33–37]. Studies performed with OmpA show that for thin membranes, an intermediate state was found in which the protein is inserted but not folded. Moreover, the distribution of the lateral stresses in the bilayer was asserted to be important since the protein is stabilized by a relatively large lateral pressure in the chain region and it was concluded that the bilayer elastic forces contribute to the thermodynamic stability of OmpA, and Hong and Tamm [38] deduce that this may be true for most integral membrane proteins.

As a concluding remark, it can be confirmed that this fluorescence anisotropy methodology can be used as a technique to probe OmpF insertion and in addition, as most membrane proteins are similar in structure to Omp's, this methodology may be used to confirm correct insertion of several different families of membrane proteins. Moreover, as the use of liposomes is becoming more popular among cell biologists, who usually are not equipped with instruments for DLS [39], this work demonstrate that fluorescence anisotropy, a widely use technique, can be a very practical and straightforward alternative to DLS to confirm membrane protein insertion.

Acknowledgments

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